Short-term nutritional folate deficiency in rats has a greater effect on choline and acetylcholine metabolism in the peripheral nervous system than in the brain, and this effect escalates with age

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Abstract

The hypothesis of this study is that a folate-deficient diet (FD) has a greater effect on cholinergic system in the peripheral nervous system than in the brain, and that this effect escalates with age. It was tested by comparing choline and acetylcholine levels in male Sprague Dawley rats fed either control or folate-deficient diets for 10 weeks, starting at age 4 weeks (the young group) or 9 months (the adult group). Folate-deficient diet consumption resulted in depletion of plasma folate in both age groups. In young folate-deficient rats, liver and lung choline levels were significantly lower than those in the respective controls. No other significant effects of FD on choline and acetylcholine metabolism were found in young rats. In adult rats, FD consumption markedly decreased choline levels in the liver, kidneys, and heart; furthermore, choline levels in the cortex and striatum were moderately elevated, although hippocampal choline levels were not affected. Acetylcholine levels were higher in the heart, cortex, and striatum but lower in the hippocampus in adult folate-deficient rats, as compared to controls. Higher acetylcholine levels in the striatum in adult folate-deficient rats were also associated with higher dopamine release in the striatal slices. Thus, both age groups showed higher cholinergic metabolic sensitivity to FD in the peripheral nervous system than in the brain. However, compensatory abilities appeared to be better in the young group, implicating the adult group as a preferred model for further investigation of folate-choline-acetylcholine interactions and their role in brain plasticity and cognitive functions.
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1. Introduction

Folate, a cofactor in one-carbon metabolism, is intimately involved in the methylation of homocysteine to methionine and S-adenosylmethionine, and it is one of the most important methyl-group donors in mammals [1-5]. Methyl-group donors are required for many methylation reactions involved in phospholipid, DNA, protein, and neurotransmitter synthesis [2,5-10]. Folate is metabolically connected to choline, another important methyl-group donor, because the synthesis of methionine from homocysteine can be accomplished by either of two enzymes: 5-methyltetrahydrofolate-homocysteine methyltransferase (which requires vitamin B_{12} and uses methyltetrahydrofolate as a methyl donor) or by betaine: homocysteine S-methyltransferase (which uses betaine, an oxidized form of choline, as a methyl donor). Choline is also used for the synthesis of a neurotransmitter (acetylcholine), for the synthesis of cell-membrane components (phospholipids), and for lipid transport (phospholipids within lipoproteins) [3,11-15]. The mechanisms by which choline metabolism is regulated have received much attention because the availability of choline can influence the synthesis and release of acetylcholine, which is involved in learning and short-term memory in animals and humans [16,17]. The correlation of clinical dementia ratings with reductions in a number of cholinergic markers, including acetylcholine levels, suggests an association between cholinergic hypofunction and cognitive deficits [18-20]. Dysregulation of the cholinergic system has been increasingly recognized as an important determinant of both cognitive decline in brain aging and age-related neurodegeneration [3,4,20-22]. Because the capacity of the adult brain to synthesize choline de novo is very low, the brain is dependent on the uptake of choline from the blood [23-27].

Both animal [4,28-33] and human [3,34-36] studies emphasize the important role of choline for physiologic integrity when sufficient levels of folate or methionine are not available in the diet. Choline- or choline-methionine-deficient diets induce severe depletion of hepatic folate in rats, suggesting that choline deficiency leads to increased use of folate to maintain hepatic methionine and S-adenosylmethionine [28-30,37]. Folate deficiency has recently been reported to reduce brain acetylcholine levels and cause abnormalities of synaptic function in vitro in mice that lack apolipoprotein E [33,38]. The metabolic relationships among folate, choline, and acetylcholine have been increasingly recognized as important because of the roles of these compounds in brain plasticity and behavior [2-4,21,39,40]. Furthermore, recent evidence has suggested that a full understanding of the metabolic relationships between these three compounds necessitates the investigation of their metabolism in the brain and peripheral tissues.

The present study was designed to test the hypothesis that a folate-deficient diet (FD) has a greater effect on choline and acetylcholine metabolism in the peripheral nervous system than in the brain, and that this effect escalates with age. The objectives for the study were as follows: (1) to analyze and compare changes in the choline and acetylcholine levels in the brain and peripheral tissues in male Sprague Dawley rats that were fed either a control or FDs for 10 weeks and (2) to determine whether there are differences in choline and acetylcholine metabolisms in response to folate deficiency between young (4 weeks old) and adult (9 months old) rats.

Folate deficiency is one of the most significant dietary health problems worldwide [1]. Poor folate status affects approximately 5% to 10% of the population exposed to folate fortification in food and more than 30% of the population without such fortification. Therefore, identifying the mechanisms that are associated with folate deficiency will be essential for targeting modifiable risk factors for cognitive decline in the elderly population.

2. Methods and materials

2.1. Materials

Reagents were obtained from Sigma Chemical Co (St Louis, Mo) and Fisher Scientific (Houston, Tex). Diet constituents, including basal mix (99%, AIN 93M) (cat# TD.09171), vitamin mix AIN-93-VX (cat# TD.94047) and folate-deficient vitamin mix (cat# TD.95052), were purchased from Harlan Laboratories, Inc (Madison, Wis). Standards for choline (cat# CF-1042) and acetylcholine (cat# CF-1043) were obtained from Bioanalytical Systems, Inc (West Lafayette, Ind). Folate standard was obtained from Sigma Aldrich (cat# F7876). Dopamine standard was purchased from Sigma (cat# H8502).

2.2. Diets

Control and FDs were prepared in the Jean Mayer United States Department of Agriculture Human Nutrition Research Center on Aging animal diet preparation kitchen by mixing 99% basal mix with 1% of an appropriate vitamin mix as previously described [31,32]. Basal mix (cat# TD.09171) contained 1% succinylsulfathiazole, a nonabsorbed sulfa drug that inhibits folate formation by gut bacteria to ensure that the animal's only source of available folate was from its diet. Diets were formulated with vitamin-free, ethanol-precipitated casein and the appropriate vitamin mix. Diets were stored at 4°C until used. The compositions of the diets are summarized in Table 1.

2.3. Animals

Young (4 weeks old, n = 10) and adult (9 months old, n = 12) male Sprague Dawley rats were obtained from Charles River Laboratories. All experiments were approved by the Animal Care and Use Committee of the Jean Mayer United States Department of Agriculture Human Nutrition Research Center on Aging at Tufts University (Boston, Mass). Animals were provided with water ad libitum and AIN-93 diet during a 1-week acclimatization period then randomly divided into 2

Table 1
Composition of diets fed to rats for 10 weeks [a]

Ingredients (g/kg diet)	99% Basal mix [b]	Dietary treatments	
		Control [c]	FD
Casein	141.41	140.00	140.00
L-Cystine	1.82	1.80	1.80
Corn starch	460.29	455.69	455.69
Maltodextrin	156.57	155.00	155.00
Sucrose	101.01	100.00	100.00
Soybean oil	40.40	40.00	40.00
Cellulose	50.51	50.00	50.00
AIN-93M, mineral mix	35.35	35.00	35.00
Choline bitartrate	2.53	2.50	2.50
TBHQ, antioxidant	0.01	0.01	0.01
Succinylsulfathiazole	10.10	10.00	10.00
Folate sufficient vitamin mix [d]	-	10.00	-
Folate deficient vitamin mix [e]	-	-	10.00

TBHQ, *tert*-Butylhydroquinone.

[a] Diets were prepared in the Jean Mayer United States Department of Agriculture Human Nutrition Research Center on Aging animal diet preparation kitchen by mixing 99% basal mix with 1% of an appropriate vitamin mix as previously described [31,32]. Dietary ingredients were supplied by Harlan Laboratories, Inc.

[b] TD.09171, basal mix (AIN-93M mix without vitamin mix).

[c] Folate sufficient diet.

[d] TD.94047, folate-sufficient vitamin mix (AIN-93 vitamin mix with folic acid).

[e] TD.95052, folate-deficient vitamin mix (AIN-93 vitamin mix without folic acid).

groups (young, n = 5 per group; adult, n = 6 per group) and maintained on the experimental diets for 10 weeks. Power analyses indicated that we required 5 rats per group to obtain an 80% chance at the .05 level of significance of detecting a difference in means of 196 pmol dopamine/mg protein, 20 nmol choline/mg tissue, and 0.7 nmol acetylcholine/mg tissue. In order to ensure comparable food intake by all groups, animals were group pair-fed. The amount of food provided to each animal was adjusted daily to ensure comparable rates of intake within age and diet groups. This procedure ensured that all age-matched animals had comparable daily consumptions of calories and nutrients. Body mass was recorded weekly. Notable observations, including all those relating to animal health, were also recorded.

2.4. Sample collection

After completion of the 10-week feeding period, the rats were fasted overnight, weighed, and euthanized by decapitation. Trunk blood was collected into Vacutainer tubes (Becton Dickinson, Rutherford, NJ) containing 0.1% EDTA. Plasma samples were separated at 2500 rpm for 15 minutes at 4°C then immediately aliquoted and stored frozen at −80°C for future analyses. After blood collection, the cortex, hippocampus, striatum, liver, kidneys, heart, and lungs of each animal were rapidly removed and weighed. The striatum from randomly chosen individual animals were used for the immediate assessment of dopamine release; the

remaining striatum and other tissues were snap-frozen in liquid nitrogen. Frozen tissues were ground to a fine powder in liquid nitrogen. Tissue powder was aliquoted and stored at −80°C until further biochemical analyses.

2.5. Blood analyses

Plasma samples (100 μL) were analyzed for glucose, albumin, total cholesterol, high-density lipoprotein (HDL), triglycerides, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and creatinine levels using an automated clinical biochemical analyzer, Olympus AU400 (Olympus, Houston, Tex). All parameters were measured using Olympus reagents. Plasma folate levels were analyzed using standard competitive immunochemiluminometric method for IMMULITE 1000 analyzer (Siemens Medical Solutions Diagnostics, Los Angeles, Calif). Plasma choline levels were measured using high-performance liquid chromatography (HPLC) system coupled to electrochemical detection as described in section 2.6.

2.6. Acetylcholine and choline analyses

Choline and acetylcholine levels were analyzed in plasma and tissue samples using HPLC system coupled to electrochemical detection as described previously [41]. Briefly, choline and acetylcholine were extracted from plasma (100 μL) and tissue (80–100 mg) samples using methanol−1 M formic acid−chloroform−water (1:0.1:2:1 by volume) solution. The supernatant fraction was collected after centrifugation at 2500 rpm for 15 minutes at 4°C, dried under a vacuum, and reconstituted in water. The content of acetylcholine and choline was measured using a Bioanalytical Systems, Inc, commercial kit consisting of acetylcholine/choline analytical column and acetylcholinesterase/choline oxidase immobilized enzyme reactor column on HPLC system coupled to electrochemical detector. Data were expressed in μmol/L for plasma samples and in nmol/g for tissue samples.

2.7. Dopamine release analyses

Dopamine release in striatal slices was assessed using HPLC system coupled to electrochemical detection as described previously [42]. Briefly, cross-cut (300 μm, McIlwain tissue chopper) slices were equilibrated in low KCl basal release buffer (21 mmol/L NaHCO$_3$, 3.4 mmol/L glucose, 1.3 mmol/L NaH$_2$PO$_4$, 1 mmol/L EGTA, 0.93 mmol/L MgCl$_2$, 127 mmol/L NaCl and 2.5 mmol/L KCl; pH 7.4) for 30 minutes at 37°C. After a 30-minute equilibration period, the buffer was replaced with a high KCL buffer (30 mmol/L KCl, 1.26 mmol/L CaCl$_2$x2H$_2$O, 57 mmol/L NaCl) in order to depolarize the neurons and evoke dopamine release, which was stimulated by 0 or 500 μmol/L of oxotremorine, a nonselective muscarinic acetylcholine receptor agonist. Samples were collected and analyzed using HPLC system (Bioanalytical Systems, Inc) coupled to

electrochemical detection as described previously [42]. The HPLC system consisted of reverse phase (C18) column (Bioanalytical Systems, Inc). The mobile phase was 100 mmol/L KH_2PO_4 buffer containing 3 mmol/L 1-heptane-sulfuric acid, 100 μmol/L EDTA, and 5.5% acetonitrile (pH 3.6) at a flow rate of 1 mL/min. Data were expressed as picomoles per milligram of protein.

2.8. Statistical analyses

The data were analyzed using 2-way analyses of variance with age group and diet as study factors using Systat version 10.0 (SPSS, Inc, Chicago, Ill). The effect of folate deficiency was judged different for young and adult rats, if the P value for the age vs diet interaction was less than .05. If the interaction P value was less than .05, Fisher least-significant-difference tests were performed [43]. All values were expressed as means ± SEM. Later in the text, we will refer to the young vs adult effect as the "age effect" and the control diet vs FD effect as the "diet effect."

3. Results

All experimental animals completed the study without any apparent health complications. The rats tolerated the diets well, with a steady increase in weight over the 10-week period. A significant difference in body mass ($P < .05$) between young and adult rats were observed, regardless of dietary folate status (Table 2). There were significant increases in cerebral cortex ($P < .01$) kidney ($P < .01$), and heart ($P < .05$) mass in adult rats as compared to those in young animals. Decreases in the ratio between tissue and whole body mass were observed in the liver ($P < .01$) and kidney ($P < .001$) in adult rats, whereas folate deficiency increased kidney-body ratio in both age groups ($P < .05$) (Table 2).

3.1. Plasma biochemistry

There were age ($P < .001$), diet ($P < .001$), and age by diet ($P < .001$) effects on plasma folate (Table 3). Folate depletion was observed in both age groups. Plasma folate levels were significantly lower (~50%) in adult rats as compared to young animals fed a control diet. However, folate levels were not different between the two age groups fed a FD (Table 3). Similar to plasma folate, choline levels were different between the 2 age groups ($P < .01$) and were further affected by folate deficiency ($P < .01$). Choline levels were not affected by folate deficiency in young rats, whereas in adult rats, plasma choline was approximately 50% higher in the folate deficient group as compared to the respective control group (Table 3). Adult rats fed a control diet showed higher glucose ($P < .01$) and lower albumin ($P < .001$) levels in plasma as compared to those in the young group. Folate deficiency significantly increased plasma glucose and albumin levels in young rats but did not affect either glucose or albumin levels in adult rats.

Table 2
Body and tissues mass of young and adult rats fed either control or folate-deficient diets

	Units	Dietary treatments	
		Control (n = 5)	FD (n = 6)
Body mass (young rats)	G	397 ± 70[a]	364 ± 10[b]
Body mass (adult rats)	g	562 ± 21[a]	566 ± 17[b]
Cortex (young rats)	mg	487 ± 41[c]	561 ± 103[d]
Cortex (adult rats)	mg	812 ± 83[c]	768 ± 42[d]
Hippocampus (young rats)	mg	49 ± 10	46 ± 5
Hippocampus (adult rats)	mg	43 ± 6	56 ± 6
Striatum (young rats)	mg	24 ± 3	17 ± 2
Striatum (adult rats)	mg	25 ± 5	28 ± 7
Liver (young rats)	mg	5865 ± 499	5977 ± 152
Liver (adult rats)	mg	5739 ± 91	5728 ± 43
Kidney (young rats)	mg	1940 ± 127	2031 ± 67[e]
Kidney (adult rats)	mg	2233 ± 55	2518 ± 13[e]
Heart (young rats)	mg	1355 ± 277	971 ± 56 [f]
Heart (adult rats)	mg	1591 ± 192	1456 ± 34[f]
Lungs (young rats)	mg	1470 ± 349	1295 ± 109
Lungs (adult rats)	mg	1817 ± 118	2033 ± 418
Cortex-body ratio (young rats)	mg/g	1.22 ± 0.09	1.52 ± 0.25
Cortex-body ratio (adult rats)	mg/g	1.44 ± 0.13	1.36 ± 0.07
Hippocampus-body ratio (young rats)	mg/g	0.12 ± 0.03	0.13 ± 0.02
Hippocampus-body ratio (adult rats)	mg/g	0.08 ± 0.01	0.10 ± 0.01
Striatum-body ratio (young rats)	mg/g	0.06 ± 0.01	0.04 ± 0.01
Striatum-body ratio (adult rats)	mg/g	0.05 ± 0.01	0.05 ± 0.01
Liver-body ratio (young rats)	mg/g	14.72 ± 1.11[g]	16.44 ± 0.21[h]
Liver-body ratio (adult rats)	mg/g	10.28 ± 0.49[g]	10.18 ± 0.36[h]
Kidney-body ratio (young rats)	mg/g	4.88 ± 0.28 [i,k]	5.59 ± 0.13[j,k]
Kidney-body ratio (adult rats)	mg/g	4.01 ± 0.24[i]	4.45 ± 0.19[j]
Heart-body ratio (young rats)	mg/g	3.35 ± 0.80	2.67 ± 0.15
Heart-body ratio (adult rats)	mg/g	2.82 ± 0.28	2.59 ± 0.11
Lungs-body ratio (young rats)	mg/g	3.71 ± 0.88	3.56 ± 0.29
Lungs-body ratio (adult rats)	mg/g	3.27 ± 0.29	3.56 ± 0.66

Data are means ± SEM. Values in the same column or row that share the same superscript letter are significantly different (analysis of variance, $P < .05$).

Total cholesterol levels were increased (~30%) in the older rats regardless of diet ($P < .05$) (Table 3). Biochemical markers of liver (ALT and AST), and kidney (blood creatinine) functions were not affected by folate deficiency regardless of age (Table 3).

3.2. Tissue choline

Choline metabolism was significantly affected by dietary folate status and by aging, as reflected in the tissue-specific choline content (Table 4). Folate deficiency effects on choline were observed in the liver ($P < .001$), kidney ($P < .001$), lungs ($P < .01$) and heart ($P < .01$); age effects were recorded in the liver ($P < .01$), kidney ($P < .01$), lungs ($P < .001$), cortex ($P < .001$), hippocampus ($P < .001$), and striatum ($P < .001$) (Table 4). There was a significant age by diet effect on choline levels in the striatum ($P < .01$), kidney ($P < .001$), and lungs ($P < .001$). Folate deficiency

Table 3

The effect of folate deficiency on plasma biochemistry in young and adult rats

Measurements	Units	Dietary treatments	
		Control (n = 5)	FD (n = 6)
Folate (young rats)	nmol/L	136.0 ± 11.0[a,f]	2.9 ± 0.4[a]
Folate (adult rats)	nmol/L	71.0 ± 5.0[b,f]	5.0 ± 0.7[b]
Choline (young rats)	μmol/L	21.0 ± 2.0	16.0 ± 2.0[g]
Choline (adult rats)	μmol/L	23.0 ± 2.0[c]	36.0 ± 2.0[c,g]
Glucose (young rats)	mmol/L	6.5 ± 0.2[d, h]	8.2 ± 0.4[d]
Glucose (adult rats)	mmol/L	7.8 ± 0.2[h]	7.7 ± 0.2
Albumin (young rats)	g/L	35.0 ± 0.2[e,i]	37.0 ± 0.6[e, j]
Albumin (adult rats)	g/L	32.0 ± 0.1[i]	33.0 ± 0.3[j]
Total cholesterol (young rats)	mmol/L	2.6 ± 0.3[k]	2.2 ± 0.2
Total cholesterol (adult rats)	mmol/L	3.0 ± 0.1[k]	2.4 ± 0.1
HDL (young rats)	mmol/L	0.9 ± 0.1	0.8 ± 0.1
HDL (adult rats)	mmol/L	1.0 ± 0.1	0.8 ± 0.1
Triglycerides (young rats)	mmol/L	1.0 ± 0.2	0.6 ± 0.2
Triglycerides (adult rats)	mmol/L	1.1 ± 0.1	1.0 ± 0.1
ALT (young rats)	U/L	62.0 ± 7.0	64.0 ± 7.0[l]
ALT (adult rats)	U/L	61.0 ± 7.0	48.0 ± 5.0[l]
AST (young rats)	U/L	349.0 ± 49.0	323.0 ± 55.0
AST (adult rats)	U/L	243.0 ± 17.0	170.0 ± 19.0
Creatinine (young rats)	μmol/L	33.7 ± 1.2	29.2 ± 1.1[m]
Creatinine (adult rats)	μmol/L	33.1 ± 2.0	30.2 ± 1.1[m]

Data are means ± SEM. Values in the same column or row that share the same superscript letter are significantly different (analysis of variance, $P < .05$).

resulted in significant decrease in choline levels in the liver and lungs of young rats (40% and 50%, respectively). Choline levels in the peripheral tissues of adult rats were even more affected by folate deficiency. Specifically, there was a significant depletion of choline in the liver (by 70%), kidney (by 50%), and heart (by 50%), whereas in the cortex and striatum, choline levels were increased as compared to

Table 4

The effect of folate deficiency on tissue choline levels in young and adult rats

Choline levels (nmol/g)	Dietary treatments	
	Control (n = 5)	FD (n = 6)
Cortex (young rats)	217 ± 19[a]	214 ± 20[b]
Cortex (adult rats)	312 ± 36[a, h]	404 ± 18[b, h]
Hippocampus (young rats)	91 ± 10	84 ± 2[c]
Hippocampus (adult rats)	114 ± 8	135 ± 10[c]
Striatum (young rats)	523 ± 25[d,i]	451 ± 22[i]
Striatum (adult rats)	325 ± 29[d,j]	403 ± 13[j]
Liver (young rats)	277 ± 32[k]	176 ± 2[e, k]
Liver (adult rats)	243 ± 15[l]	83 ± 4[e, l]
Kidneys (young rats)	428 ± 20[f]	421 ± 12
Kidneys (adult rats)	612 ± 34[f, m]	399 ± 2[m]
Lungs (young rats)	561 ± 49[g, n]	289 ± 22[n]
Lungs (adult rats)	213 ± 31[g]	284 ± 20
Heart (young rats)	86 ± 6	69 ± 4
Heart (adult rats)	79 ± 9[o]	57 ± 5[o]

Data are means ± SEM. Values in the same column or row that share the same superscript letter are significantly different (analysis of variance, $P < .05$).

Table 5

The effect of folate deficiency on tissue acetylcholine levels in young and adult rats

Acetylcholine levels (nmol/g)	Dietary treatments	
	Control (n = 5)	FD (n = 6)
Cortex (young rats)	2.42 ± 0.39[a]	2.95 ± 0.35[b]
Cortex (adult rats)	4.19 ± 0.50[a, h]	6.25 ± 0.67[b,h]
Hippocampus (young rats)	1.40 ± 0.13[c]	1.74 ± 0.29
Hippocampus (adult rats)	2.11 ± 0.08[c, I]	1.50 ± 0.09[i]
Striatum (young rats)	2.89 ± 0.2[d]	3.25 ± 0.3[e]
Striatum (adult rats)	1.42 ± 0.10[d, j]	4.51 ± 0.20[e, j]
Heart (young rats)	0.97 ± 0.05[f]	1.17 ± 0.13[g]
Heart (adult rats)	1.82 ± 0.23[f, k]	3.14 ± 0.34[g, k]

Data are means ± SEM. Values in the same column or row that share the same superscript letter are significantly different (analysis of variance, $P < .05$).

those in the respective control (by 30% and 20%, respectively) (Table 4).

3.3. Acetylcholine and dopamine

An age effect on acetylcholine was recorded in the cortex ($P < .001$) and heart ($P < .001$), whereas a folate deficiency effect was observed in the cortex ($P < .05$), striatum ($P < .001$), and heart ($P < .01$) (Table 5). There was a significant age by diet effect on acetylcholine in the striatum ($P < .001$) and heart ($P < .05$) (Table 5). Almost a 50% increase in acetylcholine levels was observed in the cortex and heart of adult rats fed a control diet as compared to levels in young rats within the same dietary group
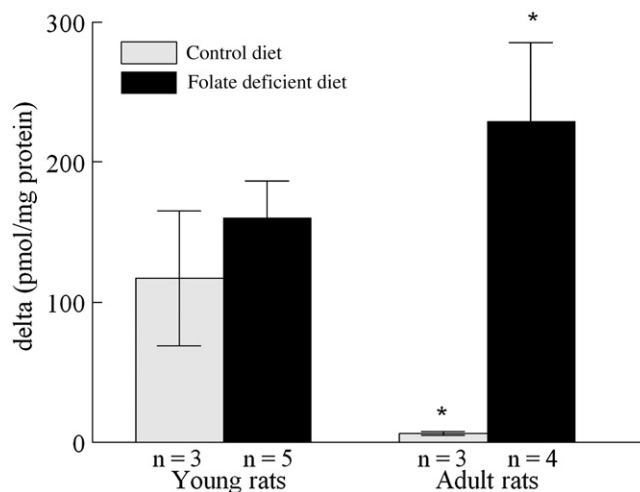

Fig. 1. The effect of folate deficiency on dopamine release was assessed in in vitro striatal slices in young and adult rats fed either control or folate-deficient diets as described previously [45]. The assay measures dopamine release at 0 μmol/L or 500 μmol/L oxotremorine (nonstimulated and stimulated dopamine release, respectively). The results expressed as delta between stimulated and non-stimulated values (delta). Data are means ± SEM. *$P < .05$ (analysis of variance).

(Table 5). These increases were further potentiated by the exposure of the adult rats to a FD. Age-related decreases in striatal acetylcholine were reversed by exposure of rats to a FD (Table 5). High levels of acetylcholine in the striatum of adult rats fed a FD were associated with significant changes in dopamine release in the striatal slices. Specifically, there was a significant increase in dopamine release in adult rats fed a FD as compared to those fed a control diet ($P < .01$) (Fig. 1). Dopamine release in young rats was not affected by folate deficiency.

4. Discussion

The present study shows that folate deprivation results in significant depletion of plasma folate in rats regardless of age. The data are consistent with our previous report of folate deficiency in young rats [31,32]. There were no visible health issues in experimental animals. Results show significant differences between the two age groups in adaptation to short-term nutritional folate deficiency as reflected by changes in choline and neurotransmitter metabolism in brain and peripheral tissues. Young rats showed more efficient adaptive responses to folate deprivation. Specifically, choline depletion was recorded only in the liver and lungs in young rats fed a FD. No other effects of folate deficiency on cholinergic neurotransmitters and tissue choline levels were recorded in young rats.

Conversely, there were significant changes in choline and acetylcholine levels and of dopamine release in adult rats exposed to folate deprivation. The depletion of plasma folate in adult rats fed a FD was associated with depletion of choline in the liver, kidney, and heart. Furthermore, adult rats showed greater choline depletion in the liver under folate deficiency than young rats. Earlier studies in young rats demonstrated a similar depletion of hepatic choline under folate deprivation, suggesting that folate deficiency negatively influenced de novo choline synthesis [29,30,37,44].

The depletion of choline in the liver (both age groups) and kidney (adult group) tissues under folate deprivation observed in this study can also be attributed to choline oxidation to betaine by choline dehydrogenase (EC 1.1.99.1) [14,15,37,43]. Earlier studies reported high activity of this enzyme in the liver and kidney [45], whereas only negligible activity was observed in the brain [46,47]. Furthermore, recent studies identified a distinct choline pool in liver and kidney tissue in mice that can be utilized under folate deprivation in combination with gamma radiation [48].

Choline is a precursor of acetylcholine [3,11,49]. Our results demonstrate a significant association between low choline levels in the heart and increased acetylcholine levels in response to folate deprivation in adult rats. Recent studies

in rats suggest a strong association between increased acetylcholine levels in the heart and the development of hypertension [50,51]. Specifically, it was suggested that the increases in acetylcholine synthesis in the heart reflect augmented parasympathetic activity counteracting or compensating for the augmented sympathetic drive of early-stage hypertension [50,51]. Our present data demonstrate that short-term nutritional folate deficiency potentiates age-related increases in acetylcholine levels in the hearts of adult rats. Furthermore, studies in rats [9,10,31,32,52,53] and humans [54-57] show that folate deficiency results in hyperhomocysteinemia, which, in turn, is associated with the risk of hypertension.

The present study also found a significant association between decreases in choline levels in peripheral tissues and increases in plasma choline concentrations in adult rats fed a FD, suggesting potential choline redistribution between tissues. These findings support studies in mice that demonstrate that choline is recycled in the liver and redistributed from kidney, lung, and intestine to liver and brain when the choline supply is attenuated [58]. Similar choline redistribution was observed in mice exposed to folate deficiency and gamma irradiation [48]. Because choline readily crosses the blood-brain barrier through an unsaturated facilitated-diffusion system, changes in plasma choline can produce parallel changes in brain choline and enhance the formation and release of acetylcholine [12,17,23,27]. A supply of choline for the synthesis of acetylcholine is essential for the normal functioning of cholinergic neurons [17,21,41]. In the initial step of acetylcholine biosynthesis, choline is taken up from the extracellular space by the sodium-dependent high affinity choline uptake system located predominantly in the terminals of cholinergic neurons [59]. Transfer of choline by sodium-dependent high affinity choline uptake system into cholinergic terminals can be the rate-limiting step for the synthesis of acetylcholine [12,17]. Furthermore, sodium-dependent choline transport is correlated with the release and on-demand synthesis of acetylcholine, which reflects the status of cholinergic neuronal activity [12,17]. Our results show that the absence of change in plasma choline levels in young rats fed a FD was associated with nonsignificant changes in choline and acetylcholine levels in evaluated brain regions as compared to levels in the respective control group. Conversely, high plasma choline levels in adult rats fed an FD were associated with changes in choline and acetylcholine content in brain regions. Specifically, there were significant increases in choline and acetylcholine levels in the cortex and striatum under folate deprivation, whereas hippocampal acetylcholine was significantly depleted. No significant changes in hippocampal choline concentrations were observed in the adult rats under folate deprivation.

Earlier studies failed to find significant changes in the enzymes that regulate acetylcholine metabolism, including choline acetyltransferase (the enzyme for acetylcholine

synthesis) and acetylcholinesterase (the acetylcholine-hydrolyzing enzyme), in several strains of rats fed folate deficient or folate-supplemented diets for 6 months [60]. Therefore, our findings of region-specific effects of folate deficiency on acetylcholine metabolism in adult rats can potentially be attributed to differential changes in the acetylcholine precursor (i.e., choline) in these brain regions.

One of the important findings of the present study is the differential effect of folate deprivation on dopamine release in the striatum with age. Our previous studies showed a significant age-related decline in striatal dopamine release [45,61,62]. The present data confirm our previous findings and show significantly lower levels of dopamine release in the striatum in the adult rats as compared to levels in young animals fed a control diet. Young rats fed an FD did not show significant changes in dopamine release, whereas in adult folate-deficient rats, there was a dramatic increase in dopamine release. Futhermore the increase in dopamine release was associated with higher levels of acetylcholine. Previous studies have demonstrated that the striatum is densely innervated by cholinergic interneurons [63-66], suggesting a potential relationship between striatal acetylcholine and dopamine release [42,66]. However, the mechanisms of this relationship remain largely unknown. Because one can assume that proper striatal functioning depends upon the precise interplay between the acetylcholine and dopamine systems, any age- or folate deficiency–related changes in this balance could subsequently involve a myriad of other neurotransmitters and neuromodulators and be translated ultimately into modification of cognitive functions.

Our results confirm the hypothesis that FD has a greater effect on the cholinergic system in the peripheral nervous system than in the brain, and that this effect escalates with age. To the best of our knowledge, this is the first comprehensive study to establish that both age groups have higher choline and acetylcholine metabolic sensitivities to short-term nutritional folate deficiency in the peripheral nervous system than they do in the brain. Furthermore, this study demonstrates that adaptation of choline and acetylcholine metabolism to folate deficiency in young rats appeared to be more efficient than in adult rats. These findings implicate adult rats as a preferred model for further investigations of molecular mechanisms of folate-choline-acetylcholine relationships.

A limitation of our study was our inability to determine whether changes in brain choline and acetylcholine metabolism result in cognitive modifications in adult folate deficient rats. Although the rat number per experimental group was sufficient to observe changes in the cholinergic system, it was underpowered for the assessment of cognitive functions, the determination of which requires 10 rats per group. Therefore, in a subsequent study, we will use a larger number of adult rats per group and conduct cognitive assessments. Furthermore, cell culture studies must be performed in the future to provide underlying mechanistic data for some of the changes observed in the present whole animal approach.

Acknowledgment

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